Cyto- and genotoxicity of dental resin composites in human gingival epithelium progenitor cells as assessed by the single cell gel electrophoresis (comet) assay

Master thesis by

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2017
Abstract

Resin composites are today amongst the most common used dental filling materials in the world. The available selection of resin composites is enormous, and unknown own brand labels are appearing on the marked in addition to the name brand composites. It is known that components from the composite materials are segregated into the oral cavity after polymerisation, but the harmful potential of these components in the concentrations achievable in the oral cavity is hitherto unknown. The aim of this thesis was to evaluate the genotoxic and cytotoxic potential of some of the most used resin composites in Norway, and compare the genotoxic potential of name brand composites with the OBL composites. The cytotoxic effects were tested with an LDH assay and the results suggest that none of the resin composites were cytotoxic. The genotoxic effects were tested with the comet assay technique and the analysis was done using the open comet imageJ plugin. The results suggest that the genotoxic potential of monomers decreases with time, and that the difference between the OBL composites and the name brand composites regarding the genotoxicity, are small. None of the composites were found to be toxic to human gingival epithelial cells. Due to methodical issues and limitations during the experiment, no definitive conclusions may be drawn from this study.
Acknowledgements

We would like to thank our main thesis advisor Hanna Tiainen. Her door was always open whenever we had questions about the research or our writing. Thank you for your patience, motivation and guidance.
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### Abbreviations and notations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>Bis-EMA</td>
<td>ethoxylated bisphenol A glycol dimethacrylate</td>
</tr>
<tr>
<td>Bis-GMA</td>
<td>bisphenol A glycidyl methacrylate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBF</td>
<td>Filtek™ Bulk Fill composite</td>
</tr>
<tr>
<td>High C</td>
<td>high control</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>Low C</td>
<td>low control</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide hydride</td>
</tr>
<tr>
<td>OBL</td>
<td>own brand label</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>TD</td>
<td>Top Dent composite</td>
</tr>
<tr>
<td>TEC</td>
<td>Tetric EvoCeram composite</td>
</tr>
<tr>
<td>TEGDMA</td>
<td>triethylene glycol dimethylmethacrylate</td>
</tr>
<tr>
<td>UDMA</td>
<td>urethane dimethacrylate</td>
</tr>
<tr>
<td>Z250</td>
<td>Filtek™ Z250 composite</td>
</tr>
</tbody>
</table>
1. Introduction

Composites were developed in the early 60’s and the light curing dental composites were introduced in the 1970's (1, 2). The early composites were deficient in several areas e.g. poor aesthetics, poor marginal adaptation and difficulty with adhesion to the enamel surface. The composite materials have gone through a major development and have unquestionably acquired a prominent place among the most used filling materials employed in direct techniques today (3). Thanks to their properties, composites have taken over the place that was occupied by amalgam, and they are now amongst the most common filling materials used to restore cavities in the permanent dentition (4).

The dental composite materials consist of two different phases: a resin matrix that polymerizes to form a solid structure and reinforcing filler particles. The resin matrix is comprised of monomers with a low molecular weight, which are converted into polymers with a high molecular weight when the filling material is exposed to light in a specific wavelength (427-491 nm) (5, 6). Most composite resins are a blend of aromatic and aliphatic dimethacrylate monomers like bisphenol A glycidyl methacrylate (Bis-GMA), triethylene glycol dimethacrylate (TEGDMA) and urethane dimethacrylate (UDMA) (6), and filler particles typically made from radiopaque glass (7). Both the monomeric composition and the filler composition have a significant effect on the qualities of the end product. While the monomers affects flexural strength, viscosity and polymerization shrinkage (8), the filler particles influences tensile strength, wear resistance and modulus of elasticity (9). The different composites available today have different areas of indication (e.g. posterior or anterior fillings) depending on their composition.

Numerous studies have indicated that various components from the composite materials are segregated into the aqueous environment of the oral cavity after polymerisation (10, 11) because of degradation processes or incomplete polymerization of the material (12). More than 30 different compounds extracted from polymerized dental composites have been detected, and amongst these, unreacted resin monomers and comonomers (13). Unreacted resin monomers remain pendant in the cross-linked polymer network and become eluted in the saliva shortly after photopolymerization, mostly within the first 24 hours (14).

Monomers have been identified as cytotoxic compounds, and the cytotoxicity of dental composites has been firmly attributed to the release of such residual monomers (15). Several studies suggest that these molecules are able to cause cytotoxic and genotoxic effects at the concentrations relevant to those released into the oral cavity (15). The underlying mechanisms of the cytotoxic effects have been well studied. The monomers are identified as
responsible for intracellular increase of the reactive oxygen species and decrease of the glutathione concentrations, which results in the DNA damage leading to cytotoxic and genotoxic effects (16, 17), apoptosis and/or necrosis (18).

Following the increasing popularity of the composite-based materials, the available selection of resin-based composites has exploded. Today, there are numerous manufacturers producing a great variety of resin-based composites. In addition, unknown own brand label (OBL) composite materials are appearing in public tenders, often to a lower and competitive price. The scientific literature on these OBLs is deficient and several studies have suggested that more research is needed on the OBLs to secure that they meet prevailing standards (19).

The aim of this study was to determine the genotoxic potential of widely used resin composites in the public dental healthcare in Norway and compare the genotoxic potential of OBLs versus name brand composites. In order to establish if the monomers activity changes with time, the monomers genotoxic potential were tested at different time points. The null hypothesis was that there is no significant difference in the potential to cause DNA damage when comparing OBL composites with name brand composites.
2. Materials and methods

2.1 Materials used in the study

Five different composite resin materials were used in the present study: three name brand composites and two corresponding own brand label composites. Detailed information on the composition of the composite materials and the manufacturers are given in Table 1. The chosen composites are a selection from the most used composite materials in the public dental healthcare in Norway 2015 (20).

Table 1: Dental composites used in this study

<table>
<thead>
<tr>
<th>Material</th>
<th>Type and colour</th>
<th>Manufacturer</th>
<th>Organic matrix</th>
<th>Inorganic filler</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtek Z250 (Z250)</td>
<td>Microhybrid</td>
<td>3M ESPE</td>
<td>Bis-GMA1-5% UDMA5-10% Bis-EMA5-10% TEGDMA 1-5%</td>
<td>ZrO₂-SiO₂ cluster</td>
</tr>
<tr>
<td>Tetric EvoCeram (TEC)</td>
<td>Nano-hybrid</td>
<td>Ivoclar Vivident</td>
<td>UDMA Bis-GMA Bis-EMA Decandiol Dimethacrylate</td>
<td>Barium glass Ba-AL-fluorosilicate glass Ytterbium trifluoride Highly dispersed SiO₂ Prepolymerized filler (PPF)</td>
</tr>
<tr>
<td>Filtek Bulk Fill (FBF)</td>
<td>Bulk fill</td>
<td>3M ESPE</td>
<td>Bis-GMA UDMA BisEMA</td>
<td>ZrO₂, SiO₂</td>
</tr>
<tr>
<td>4U (OBL)</td>
<td>Nanohybrid with fluoride/micro-hybrid</td>
<td>Nordenta</td>
<td>Bis-GMA Mixture of poly- and difunctional methacrylates</td>
<td>Barium glass and fused silica</td>
</tr>
<tr>
<td>Top Dent (TD; OBL)</td>
<td>Nano hybrid / micro hybrid</td>
<td>DAB Dental</td>
<td>TEGDMA 1-5% Bis-GMA 1-10% UDMA 1-10% TMPTMA&lt;1%</td>
<td>No information provided</td>
</tr>
</tbody>
</table>

OBL: own brand label
Bis-GMA: bisphenol A-glycidyl methacrylate
Bis-EMA: ethoxylated bisphenol A glycol dimethacrylate
UDMA: urethane dimethacrylate
TEGDMA: triethylene glycol dimethacrylate
2.2 Preparation of test specimens

The test specimens were fabricated under aseptic conditions according to the manufacturers’ directions and the ISO standard recommendations. Custom-made moulds were used to ensure that all test specimens were made with equal volume, size correspondent to an average sized filling. The same curing lamp (Elipar™ DeepCure-L, 3M ESPE, Neuss, Germany) was used to cure all the resin blocks, the distance between the curing light and the specimens were kept equal for all specimens and the polymerization were initiated from the top, simulating clinical situations.

2.3 Preparation of the extraction medium

Under healthy conditions, adults will produce approximately 500-1500 ml saliva per day (21). The mean total surface area of the mouth is 214 cm² with no difference due to gender (22). Approximately 20% of the total surface area is tooth surfaces (22), and the surface area of one tooth therefore equals 1.3 cm². An average person therefore produces 6.25 ml saliva/day per tooth. The ratio between the volume of the extraction medium (CnT-24 gingival epithelial culture medium) and the surface area of the test specimens was therefore set to 6.25 ml/1.3 cm². The resin specimens were soaked in the cell medium for 1, 3, 7 and 21 days. The conditioned cell culture media were then used to assess the genotoxicity of the eluted monomers.

2.4 Cell culture

Normal human gingival epithelium progenitor cells (HGEPs.15, CELLnTEC, Bern, Switzerland) were used as a model for the biological response of cells towards the residual monomers leached out from the cured composites. The cells were cultured in a 24-well tissue culture plate in CnT-24 gingival epithelial culture medium at 37°C in a humidified atmosphere of 5% CO₂ until 70% confluence was reached. Cells were then incubated in the conditioned extraction media for 24 h at 37°C in a humidified atmosphere of 5% CO₂ (n = 5 per group).

2.5 Genotoxicity assay

An alkaline comet assay was used to quantify permanent DNA damage in HGEPs.15 cells following the exposure to the conditioned extraction media. Cells were detached from the well plates using trypsin/EDTA, resuspended in 1.2 ml culture medium and immediately put on ice. After centrifugation (5 min at 0.2 G), the supernatant was removed and the cell pellet was mixed in 44 µl low melting point agarose. The agarose-cell suspension was divided into two equal drops of 20 µl and placed on an agarose coated microscope slide. Slides were stored at 4°C for 10 min for complete gelation. The high control slides were placed in slide holder filled with cold 3% H₂O₂ for 5 min. All slides were then placed in slide holders filled
with a cold lysis solution (2.5 M NaCl, 0.1M EDTA, 10 mM Tris-HCl, pH 10, 1% triton X-100) and stored at 4°C for 1 h. All slides were then placed in alkaline electrophoresis solution (0.3 M NaOH, 1mM EDTA) for 20 min at 4°C for DNA unwinding before electrophoresis was run for 30 min at 25 V and 350 mA. Slides were neutralised with cold PBS and d-H2O for 10 min each and dried overnight at RT.

Nuclei were stained with SYBR gold (Thermo Scientific, Waltham, MA, USA) at RT for 15 min in darkness shortly before being imaged. Slides were analysed using a fluorescence microscope (DM6000 FS, Leica, Wetzland, Germany). At least 50 nuclei per droplet (100 per sample) were imaged and analysed using the OpenComet plugin for ImageJ (v1.3). The samples were originally imaged with the same objective at 10x magnification. Results are given as %DNA in tail relative to the low control (cells cultured in unconditioned medium) and high control (cells treated with 3% H2O2).

2.6 Cytotoxicity assay

Lactate dehydrogenase (LDH) activity was measured as an indicator for membrane related cell death in cells exposed to the 1 d conditioned extraction medium. 100 µl of cell culture medium was collected after 24 h and mixed 1:1 with a reaction mixture according to manufacturer instructions (Cytotoxicity detection kit, Roche Diagnostics, Mannheim, Germany) and incubated at RT for 30 min. The oxidation of NADH was measured spectrophotometrically (ELx800, BioTek Instruments, VT, USA) at 490 nm. Results for the tested groups are given relative to a low control (cells cultured in unconditioned medium) and a high control (cells cultured in unconditioned medium with 1% triton X-100). The cytotoxicity of test samples is presented relative to high and low control:

\[
Cytotoxicity (\%) = \frac{\text{sample} - \text{low control}}{\text{high control} - \text{low control}} \times 100
\]

2.7 Statistical analysis

Statistical analyses were performed using the statistical software SigmaPlot 13.0 (Systat Software, San Jose, USA). All tests were performed at a confidence level of 95% and post hoc retrospective power analyses were performed to find the statistical power of the tests (alpha = 0.050). Statistical comparison of the different groups was performed using one-way analysis of variance (ANOVA) test followed by post hoc tests for pairwise comparisons performed using Holm-Sidak test. Statistical significance was considered at a probability p < 0.05.
3. Results

3.1 Genotoxicity

Genotoxicity was measured with an alkaline comet assay, which assesses the amount of DNA damage caused by the monomers eluted to the conditioned extraction medium. The results of the comet assay are shown in Figure 1. The results suggest that the highest DNA damage occurs in cells exposed to the media conditioned for one day and the least amount of damage occurs in cells exposed to the media conditioned for day 21. The exception is for TEC and TD, where the highest DNA damage occurs at day three. Results for 4U day 3 are missing because of an unknown error resulting in no detectable comets.

![Figure 1: DNA damage caused when human gingival epithelial precursor cells were exposed to cell culture media exposed to the cured composited for up to 21 d. *p < 0.05 in comparison to corresponding low control value](image)

Electrophoresis at high pH results in structures resembling comets when observed with fluorescence microscopy, where intact DNA is the comet head and fragmented DNA in the tail. The intensity of the comet tail relative to the head reflects the number of DNA breaks. Followed by an analysis, the comets are used to determine the extent of DNA damage.
The appearance of representative comets for each of the test groups and time points are shown in Figure 2 to Figure 5. The intensity profiles and outlines of all the comets and comet heads, automatically given by the open comet plugin, are shown as an overlay in each image.

The outlines of the comets are colour coded, and can be interpreted as follows:

- Red outline and number: Normal comets
- Yellow outline and number: Outlier comet (e.g. Figure 4, Z250 day 7)
- Gray outline: Removed shape (e.g. Figure 6B)

The comets that are clustered or at the edges of the slides are not considered for analysis. The comets profile analysis is also colour coded (Figure 6A).

- Green profile: Comet head
- Yellow profile: Comet tail
- Blue profile: Comet (equal to head plus tail)

TD day 2 generally has comets with longer tails than the other groups for cell media conditioned for 3 days (Figure 3), which corresponds to the results of the analysis represented in Figure 1.

Figure 2 – Figure 5 show a representative view of the comets for each sample to illustrate the comet shapes and their analysis and are therefore not in scale with each other.
Figure 2: Representative comets detected by OpenComet for cell media conditioned for 1 d.
Figure 3: Representative comets detected by OpenComet for cell media conditioned for 3 d.
Figure 4: Representative comets detected by OpenComet for cell media conditioned for 7 d.
Figure 5: Representative comets detected by OpenComet for cell media conditioned for 21 d
Figure 6: Possible errors in comet analysis. (A) Comet not analysed correctly by the OpenComet plugin, (B) Perfect comet not analysed by the OpenComet plugin
3.2 Cytotoxicity

Cytotoxicity was tested with an LDH assay. The results of the cytotoxicity test are shown in Figure 7. There was no statistical difference between the test groups and the low control ($p > 0.05$). The cytotoxicity for all of composite materials was almost at zero or below zero. It should be noted that less than 30% cell death is considered as non-cytotoxic.

Figure 7: LDH activity measured from the cell culture medium of cells exposed to extraction medium conditioned for 1 d. Low C: unconditioned medium, high C: unconditioned medium supplemented with 1% triton-X-100.
4. Discussion

4.1 Cytotoxicity

To test the cytotoxicity, an LDH assay was performed. The LDH assay is a way to biochemically quantify destroyed cellular structures (23). Lactate dehydrogenase (LDH) is released when the cell membrane is disrupted (23). A disruption of the cell membrane usually correlates with an increase in cell death, which is why the activity of LDH detected in the cell culture supernatant can be used as a quantitative measurement of cell death in vitro (24). The cytotoxic effect of the monomer exposure was tested with the medium after 24h exposure, as the majority of the leaching is complete within the first 24 h (25). Overall, the data from the LDH assay suggests that none of the composites were cytotoxic. After 24 h, all of the composites were below the maximum accepted threshold of 30% cytotoxicity of medical devices according to the ISO-10993:5 for all materials. Even though the in vitro testing of the cytotoxicity does not replicate the clinical situation, the results serve as mean to compare the different materials. There was no statistical significant difference either between the different composites or the composites in comparison to the low control.

4.2 Genotoxicity

The comet assay, which was used in this study, is a simple and reliable method for studying DNA damage (26). The main principle is that cells are embedded in agarose and lysed, followed by alkaline electrophoresis to unwind and denature the DNA and reveal breaks in the DNA strands (26). It is useful in detecting the genotoxicity of the eluted monomers because of the availability of data at the individual cell level and the need for a relatively low number of cells per sample (26). The results from the comet assay suggest than the amount of DNA damage is highest at day one, with the exception of the TD composite which had the most damage at day 3. The general trend is that the percent of DNA damage decreases with time with time, the lowest amount of damage was detected at day 7 and 21.

After 24 h, the Z250 had the highest amount of DNA damage. Johnsen et al. found that the total amount of monomer elution after 24 h in acetone ranked according from the highest to the lowest amount is 4U > TD > Z250 (19). According to these findings, it is expected that the Z250 would cause the lowest amount of DNA damage and 4U the highest amount of DNA damage at day 1. The results in this study suggest the opposite. The OBL composites resulted in less DNA damage than Z250 at day 1 and 7. The 4U caused the highest amount of cell damage of all the tested composites at day 7, but less damage than Z250 at day 1 and 21. There were not enough comets in the 4U day 3 sample to quantify the DNA damage, which means that it is impossible to say whether this sample has a similar trend to Z250 and FBF or behaves more like TEC and TD, which showed highest damage on day 3.
For day 7 and 21, all of the tested specimens resulted in significantly less DNA damage than measured in the low control at the same time points, something that is not expected and gives reason to question the results. Similar or higher amount of DNA damage is expected in the exposed cells at any time point, not less damage than in the low control. Another reason to question the validity of the results is the lack of a high control.

\[ \text{H}_2\text{O}_2 \] is a very reactive and powerful oxidant, and is know to cause DNA damage (27). The cells in the high control were exposed to \[ \text{H}_2\text{O}_2 \] to purposefully cause a great amount of DNA damage. The genotoxicity analysis should have shown DNA damage close to 100% DNA in tail. Instead the high control group did not show significantly higher values than the control group. Therefore, the high control could not be used and the %DNA in tail for the test groups (Figure 1) could not be shown relative to the low and high control as shown for LDH activity (Figure 7). Only the result of Z250 day 1, TEC day 21, 4U day 21 and TD day 2 and 21 were found statistically significant in comparison to the low control value.

That the amount of DNA damage for all composites were slightly lower than the low C at day 7 and 21 may be a result of how the monomers activity changes with time. The exposed medium is stored in the fridge for 21 days during which the eluted monomers can react with different components in the media. This can influence the monomers reactivity over time, and hence affect the interaction between the monomers and the cells, which mean some differences can be expected. In the absence of a high control group it is difficult to say if this may be the cause of the low values at this time.

4.3 Methodical issues

There are several sources of error and methodical limitations to this study. The OpenComet plugin automatically detects comets for the analysis. The selected comets have to be controlled manually to make sure that invalid and outlier comets are excluded. In addition, some correct comets were not analyzed at all because the program would not allow to manually add selection to the automated comet detection. Even though two operators controlled all the comets, some invalid shapes may have made it through the data collection (Figure 6). This should not be significant due to the amount of comets analysed and the statistical calculations, but a small influence cannot be excluded. For the purpose of the analysis, the following comets were discarded: superimposed comets, irregular comets, debris and comets without a distinct shape (i.e. clouds).

The pictures had to be manually taken for each slide in the microscope. Because one picture can only show a small part of the slide, it manually had to be moved to different parts of the microscopic slide to get at least 50 comets per slide. The result could be that the same
comet could be in more than one picture, and could potentially be accounted for more than one time.

The OpenComet calculates and outputs many parameters concerning the comet, allowing the user to choose the desired one for further analysis (26). The extent of DNA damage is related to the amount of DNA in tail and three measures of DNA have been shown particularly good indicators for the underlying damage: DNA percent in tail, tail moment and Olive moment, where the olive moment is considered to be the most reliable measure (28). In this study the DNA percent in tail was the only used parameter. The tail moment and Olive moment also takes into account the tail shape, so they tell a bit more about the extent of the DNA damage. But the lack of high control would still have been a big source of error even when analysing the different comet parameters.

4.4 Limitations

When the resin restorations are cured, the surface layer is exposed to air during polymerization. The presence of oxygen throughout light irradiation results in formation of a resin-rich inhibited layer at the uppermost surface of the resin composite (29). These unreacted components may become eluted into saliva and might result in adverse effects. The significance of removing the leaching monomers from the oxygen inhibited layer has been documented in the literature (30). In a clinical situation the oxygen-inhibited layer is removed by the dentist, who polishes the accessible surface of the filling. In this study, the surfaces of the resin blocks were not polished. This may contribute to a higher leakage of monomers than in a clinical situation.

It is difficult to recreate the conditions in the mouth. In this study, it was tested whether the substances released by resin-based dental restorative materials were able to cause cytotoxic or genotoxic effects at concentrations relevant to those released into the oral cavity, as suggested by other studies (15). It is difficult to recreate the conditions in the oral cavity in vitro studies. Because of the continuously saliva flow, swallowing, eating and the complex composition of saliva, which also varies from one person to another, it is difficult to predict the concentration of monomers in vivo and how the monomers will interact with the epithelial cells.
5. Conclusion

The main finding in this study is that there is the most DNA damage at day one, and least DNA damage at day 21. However, there was no high control group to compare the results to, and therefore no definitive conclusion can be drawn from this study regarding cytotoxicity and genotoxicity. Sources of error need to be eliminated, and the experiment must be repeated in order to obtain reliable results. None of the composites were found to be toxic to human gingival epithelial cells.
6. References